Available online at www.sciencedirect.com

ScienceDirect

Virology 374 (2008) 322–337

VIROLOGY

www.elsevier.com/locate/yviro

Replicating adenovirus HIV/SIV recombinant priming alone or in combination with a gp140 protein boost results in significant control of viremia following a $SHIV_{89.6P}$ challenge in *Mamu-A*01* negative rhesus macaques

L. Jean Patterson [a], Jennifer Beal [a], Thorsten Demberg [a], Ruth H. Florese [a], Nina Malkevich [a], David Venzon [b], Kris Aldrich [a], Ersell Richardson [a], V.S. Kalyanaraman [c], Irene Kalisz [c], Eun Mi Lee [c], David C. Montefiori [d], Frank A. Robey [e], Marjorie Robert-Guroff [a,*]

[a] *Vaccine Branch, National Cancer Institute, Bethesda, MD 20892, USA*
[b] *Biostatistics and Data Management Section, National Cancer Institute, Bethesda, MD 20892, USA*
[c] *Advanced BioScience Laboratories, Inc., Kensington, MD 20895, USA*
[d] *Duke University Medical Center, Durham NC, USA*
[e] *AriaVax, Inc., Gaithersburg, MD 20850, USA*

Received 2 October 2007; returned to author for revision 13 November 2007; accepted 24 December 2007
Available online 5 February 2008

Abstract

Previously, replicating adenovirus type 5 host range (Ad5hr)-HIV/SIV recombinant priming in combination with SIV envelope boosting, resulted in significant, durable protection in 39% of rhesus macaques after SIV_{mac251} challenge. Both Env-specific antibody mediating ADCC, and cellular immunity correlated with protection. Here we evaluate the relative immunogenicities of novel HIV proteins and their contribution to protection in a $SHIV_{89.6P}$ model. All groups were primed with Ad-HIV$env_{89.6P}$, SIVgag_{239}, and SIVnef_{239} recombinants. One group was not boosted, one received $HIV_{89.6P}$gp140ΔCFI protein, and one a novel HIV-1 poly-peptide "peptomer". The $HIV_{89.6P}$gp140ΔCFI protein in adjuvant strongly boosted Env-specific antibody and memory T cell responses in blood and tissue, resulting in significant reductions in acute and set point viremia. Macaques not boosted, showed a significant reduction in set point viremia, a full 32 weeks after the last Ad priming immunization. The HIV peptomer-boosted group showed a trend toward chronic viremia reduction, but was not protected.

Keywords: Replicating adenovirus vectors; prime/boost; HIV/SIV vaccines; $SHIV_{89.6P}$ challenge; Rhesus macaques

Introduction

Despite decades of research aimed at developing an effective HIV vaccine, the AIDS epidemic continues unabated. Among the current candidates that are being evaluated, ones utilizing viral vectors to deliver HIV genes to the immune system are of most interest. Both replication-competent and incompetent adenovirus (Ad) recombinant vaccine strategies are currently being pursued. They are already known to exhibit protective efficacy in preclinical animal trials (Buge et al., 1997; Demberg et al., 2007; Letvin et al., 2004; Liang et al., 2005; Malkevitch et al., 2006; Patterson et al., 2004; Zhao et al., 2003a), as well as proving to be safe and immunogenic in people (Catanzaro et al., 2006). Recent trials involving the use of Merck's Ad5 trivalent vaccine however, failed to show protective efficacy in two separate cohorts of volunteers and were halted. Nevertheless, additional Ad-based vaccines are in development and improvements to these vectors should increase chances of future success.

An attractive feature of Ad vectors is that, depending on the strain, they preferentially infect epithelial cells which line the upper respiratory, gastrointestinal, and reproductive tracts. Ninety percent of all HIV infections are acquired at mucosal

* Corresponding author. NIH, NCI, 41 Medlars Drive, Building 41, Room D804, Bethesda, MD 20892-5065, USA. Fax: +1 301 402 0055.
E-mail address: guroffm@mail.nih.gov (M. Robert-Guroff).

0042-6822/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.12.037

surfaces (Duerr et al., 2006) and in turn, the virus initially targets $CD4^+$ memory T cells which reside at these same sites (Li et al., 2005b; Mattapallil et al., 2005; Veazey et al., 1998, 2000). Therefore, priming and expanding immune cells responsible for blocking initial infection at the mucosa are vital.

Our lab has been developing and characterizing replicating Ad-HIV/SIV recombinant vectors expressing multiple inserted genes as immunogens in chimpanzees and rhesus macaques. Historically, we have shown that our vectors can prime potent cellular, humoral and mucosal immunity and in combination with a protein boost, can further expand the breadth of the immune response to protect from homologous and heterologous HIV challenges of chimpanzees (Lubeck et al., 1997; Robert-Guroff et al., 1998; Zolla-Pazner et al., 1998) and from virulent SIV_{mac251} and $SHIV_{89.6P}$ challenges in rhesus macaques (Demberg et al., 2007; Malkevitch et al., 2006; Patterson et al., 2004; Zhao et al., 2003a). Immune correlates of protection included Env-specific cellular immunity, and Env-specific binding antibody which mediated antibody-dependent cellular cytotoxicity (ADCC) (Gomez-Roman et al., 2005) but not conventional *in vitro* neutralization of the challenge virus. Importantly, it was additionally shown in chimpanzees by Peng, *et al.* that Ad-HIV*env* recombinant priming followed by an HIV_{SF162} gp140ΔV2 protein boost effectively elicited neutralizing antibodies to primary HIV isolates (Peng et al., 2005) and broadly reactive antibodies capable of mediating ADCC across clades (Gomez-Roman et al., 2006a,b).

The objective of the current study was to dissect the contribution of a protein boost to protection as well as assess the relative immunogenicities of novel HIV proteins. To accomplish this, we shifted to a $SHIV_{89.6P}$ challenge model in *Mamu-A*01*-negative rhesus macaques and further evaluated immune responses in multiple tissue compartments in order to identify correlates of protection.

Previously, we were able to achieve dramatic protection in a subset of rhesus macaques that cleared viremia or remained aviremic following Ad type 5 host-range mutant (Ad5hr)-SIV*env/rev* priming coupled with an SIV "peptomer" boost (Malkevitch et al., 2006; Patterson et al., 2004). The peptomer, initially formulated based on the HIV_{MN} sequence (Robey et al., 1995, 1996), represents 18 amino acids located within part of the CD4 binding site on gp120. When this linear peptide is linked end to end, it acquires a secondary structure containing α-helical and β-sheet conformation, proposed to be structurally similar to the envelope on the surface of the virion. The peptomer is capable of binding CD4. The site targeted by the peptomer is composed of the β20–β21 component of the bridging sheet that connects the inner with the outer domains of gp120. This component is highly conserved across HIV and SIV strains, and contains both B and T cell epitopes. Here we used a $HIV_{89.6P}$ peptomer as boosting immunogen, not only because HIV is more relevant than SIV to people, but to determine if the potent protection observed in macaques boosted with SIV peptomer would translate to the SHIV system.

Second, we used a homologous prime-boost approach consisting of Ad5hr-$HIV_{89.6P}$gp140ΔCFI priming and boosting with $HIV_{89.6P}$gp140ΔCFI expressed protein. Letvin and colleagues previously showed that the genetically modified $HIV_{89.6P}$*env* gene containing deletions of the cleavage site, fusion peptide and interhelical domains was able to broaden antibody responses to Env (Mascola et al., 2005), and when incorporated into both DNA and a replication defective Ad5 vector, resulted in protection from a $SHIV_{89.6P}$ challenge in rhesus macaques (Letvin et al., 2004). We hypothesized that when expressed as a protein, this immunogen, following homologous priming with replicating Ad5hr-$HIV_{89.6P}$-p140ΔCFI, would elicit protective antibody responses.

Finally, we also included a vaccine arm which was not boosted, only primed with Ad recombinants, in order to clearly evaluate the relative contribution of the Ad priming immunizations and protein boosts to immunogenicity and protective efficacy. Prior to this study we had not tested the protective role of recombinant priming alone in either SIV or SHIV rhesus macaque models.

Lastly in retrospect, groundbreaking vaccine studies published a few years ago which made use of the $SHIV_{89.6P}$ virus challenge (Amara et al., 2001; Rose et al., 2001; Shiver et al., 2002) included macaques that were positive for a MHC class I allele, *Mamu-A*01*, known now to mediate natural control of viral infection in part due to a dominant $CD8^+$ T cell response (Mothe et al., 2003; Zhang et al., 2002). Since then, only a handful of studies have controlled for this variable in subsequent vaccine studies by excluding them from immunization groups (Demberg et al., 2007; Letvin et al., 2004; Liang et al., 2005). While useful for *in vivo* monitoring of immunity against dominant epitopes such as Gag p11c, *Mamu-A*01*-restricted responses represent only a fraction of the complex immunological response to the myriad of epitopes included in a vaccine. Consequently, with regard to both immunogenicity and protective efficacy, accurate comparison of vaccine modalities can only be accomplished with exclusive use of *Mamu-A*01* negative macaques.

Therefore, our goal here was to test the ability of novel boosting immunogens to elicit humoral and cellular immunity in *Mamu-A*01* negative rhesus macaques and to compare protective efficacy of these vaccine regimens, including Ad-recombinant priming alone, among the immunization groups.

Results

Adenovirus recombinant priming elicits both HIV-Env and SIV-Gag specific ELISpot responses which are boosted and maintained following $HIV_{89.6P}$ gp140 protein immunization

As summarized in Materials and methods, three groups of six monkeys each were mucosally primed with replicating Ad5hr-HIV/SIV recombinants containing HIV$env_{89.6P}$gp140ΔCFI, SIVgag_{239}, or SIVnef_{239}. One group received no boost, the other two were boosted at weeks 24 and 36 with either $HIV_{89.6P}$ gp140ΔCFI envelope protein or $HIV_{89.6P}$ peptomer. Six control monkeys received empty replicating Ad5hr vector, and half were boosted with adjuvant alone or PBS.

Virus-specific IFN-γ^+ ELISpot responses using freshly isolated lymphocytes from PBMC were measured throughout the

course of immunization. Prior to boosting, all immunized animals received identical Ad recombinant immunogens and responses were averaged for ease in presentation. After one immunization with replicating Ad recombinants, only low level HIV Env- (Fig. 1A) and SIV Gag- (Fig. 1B) specific responses were detectable 2 and 10 weeks later. A second priming immunization, however, boosted both Env- and Gag-specific responses. Env-specific responses were significantly higher compared to the control group both at week 14 ($p<0.0001$) and week 22 ($p<0.0001$).

After the first immunization with gp140, Env responses were significantly boosted ($p<0.0001$), peaking to an average of 332 spots and were maintained at a similar level up to the time of challenge at week 44 (Fig. 1A). Moreover, throughout the entire boosting period (weeks 26 – 44), the gp140 group exhibited significantly higher Env ELISpots compared to non-boosted ($p=0.0026$) and peptomer boosted ($p=0.012$) groups. In fact, at all time points measured, the Env-specific responses in the gp140 boosted group were significantly different from controls with p values of 0.0022, 0.0087, 0.0044, and 0.0022 at weeks 26, 34, 38 and 44 respectively. Env-specific ELISpot responses for the non-boosted and peptomer boosted macaques were significantly elevated above control values at week 26, possibly due to continued presentation following the replicating vector prime, however, these differences were not manifested at later times during the immunization course.

Two priming immunizations with Ad recombinants were also required to elicit a significant response to SIV Gag for the immunized macaques compared to controls at week 14 ($p=0.0003$) and week 22 ($p=0.0013$), as seen in Fig. 1B. Interestingly, each subsequent boost with gp140 was associated with elevated SIV Gag-specific ELISpot responses that were significant versus controls at the level of $p=0.0022$ and $p=0.017$ at weeks 26 and 38, and over the entire boosting interval in comparison to both the control ($p=0.0072$) and peptomer boosted macaques ($p=0.014$). We have previously observed this phenomenon (Zhao et al., 2003a; Patterson et al., 2003) and attribute it to either an adjuvant effect, since here only the gp140 group received MPL-SE, or to

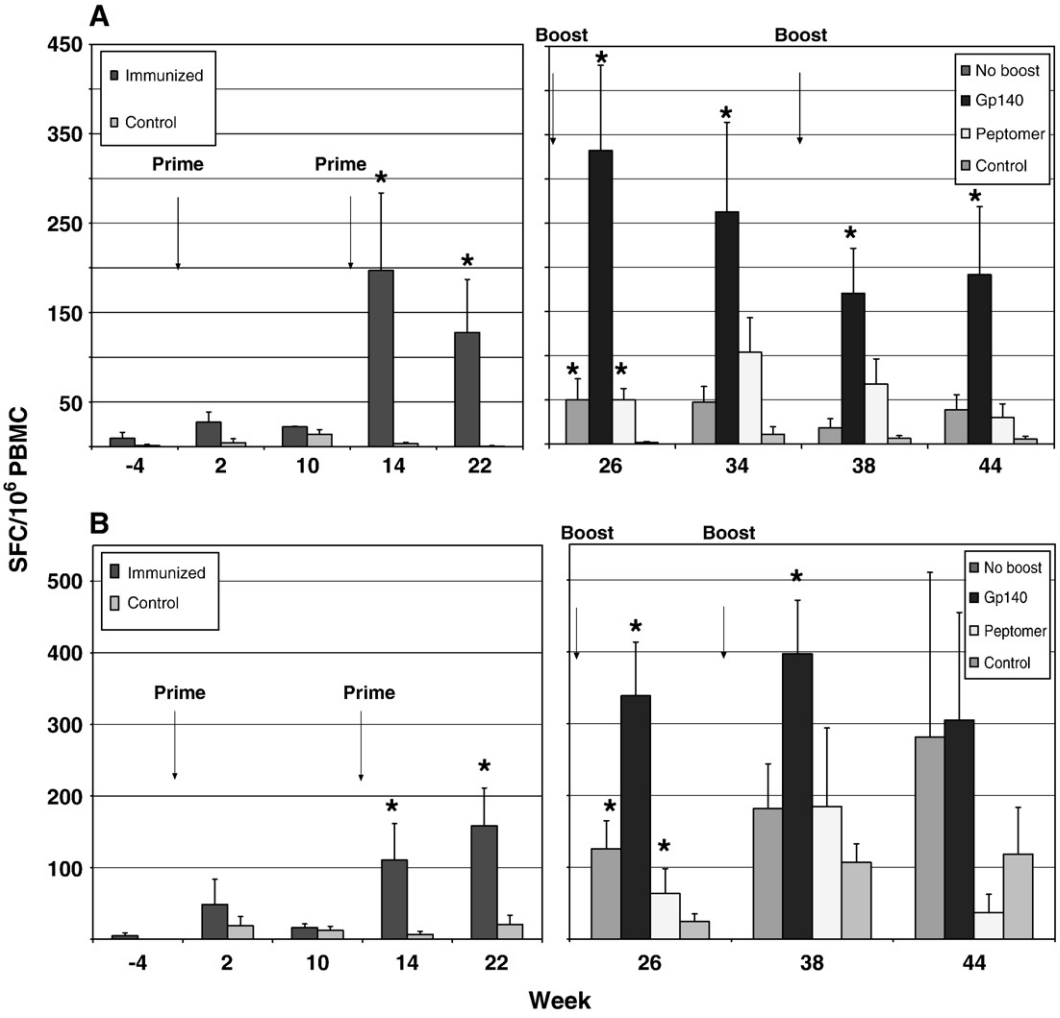

Fig. 1. ELISpot values in response to stimulation with HIV $Env_{89.6P}$ (A) or SIV Gag_{239} (B) overlapping peptide pools over the entire immunization course. Prior to week 26, groups I, II and III were identical and responses therefore were averaged to represent the entire immunized group. After boosting, individual groups are shown separately and designated as no boost, gp140, peptomer, or control. Group means showing the number of spots per million PBMCs for each macaque are shown with standard error of the mean (sem) bars given. Data from the week 34 time point reflecting SIV Gag responses for all macaques were unavailable due to technical difficulties. An asterisk denotes a significant difference in comparison to the control response for that time point. Associated p values are provided in the text.

Table 1
Peak virus-specific T cell proliferative responses

Group	Env 89.6P	AT-2 89.6P	SIV Gag	SIV Nef	Peptomer
Post priming					
Immunized	8.1±1.7	7.6±1.4	4.3±0.5	2.3±0.5	1.9±0.2
Control	0.9±0.1	2.0±0.5	1.5±0.4	3.0±1.1	2.1±0.5
Post boosting					
No boost	5.9±2.3	6.8±1.8	2.7±0.6	6.9±2.1	3.0±0.9
Gp140	7.1±1.6	8.8±2.3	1.8±0.4	4.6±2.6	3.2±0.7
Peptomer	6.4±1.8	10.4±1.4	5.5±1.6	4.8±2.3	6.3±1.2
Control	0.7±0.2	1.7±0.5	1.6±0.3	1.7±0.6	2.6±0.6

non-specific bystander activation, since the protein might be providing a general $CD4^+$ T helper response. Elevated Gag responses in comparison to control levels were not detectable, however, for any immunization group at challenge (week 44) at which time intra-group values were highly variable. As with the Env-specific responses, Gag-specific responses were significantly different from controls for both the non-boosted ($p=0.026$) and peptomer boosted ($p=0.015$) groups only at week 26.

Potent HIV Env-specific T cell proliferative responses are induced after Ad priming, and sustained over the boosting period for all macaques

Peak proliferative responses over the priming and boosting phases of immunization for all antigens evaluated are shown in Table 1. Additionally, Env-specific responses tested at each time point following stimulation with gp140 protein, are shown in Fig. 2. Similar to the ELISpot profile, a second Ad immunization was required to induce significant proliferative responses for the immunized macaques versus controls at a level of $p<0.0001$ for both weeks 14 and 22 (Fig. 2). At week 26, two weeks after the first protein boost, groups boosted with HIV peptomer or gp140 exhibited significantly greater Env-specific responses compared to controls ($p=0.0087$ and $p=0.0065$, respectively), with the gp140 boosted group additionally exhibiting significantly greater responses than the non-boosted group ($p=0.0065$). At all subsequent time points, however, the non-boosted group showed increasingly higher Env-specific responses, with differences in comparison to the controls actually peaking at the time of challenge ($p=0.0043$ for weeks 34, 38 and 44). The slow, gradual acquisition of proliferative responses to Env in this non-boosted group could be an as-yet-unobserved feature of replicating Ad vectors, either due to the continued presentation of antigen to the immune system or via a unique mechanism of presentation. In addition, the gp140 boosted macaques continued to maintain responses over weeks 34 to 44 significantly greater than control levels ($p=0.0043$ for each). The peptomer group also exhibited significant proliferative responses in comparison to the control group ($p=0.0043$ at weeks 34 and 44).

Peak responses were similar across groups when aldrithiol-2 (AT-2) inactivated $SHIV_{89.6P}$ virions were used to stimulate PBMC (Table 1). Moreover, during priming and boosting, all groups continued to exhibit significantly higher responses at all time points versus controls ($p<0.026$ for no boost group, $p<0.0087$ for gp140 group, and $p<0.0043$ for peptomer boosted group) except at the time of challenge, when only peptomer boosted macaques were comparatively higher ($p<0.0043$) (data not shown).

Proliferation in response to SIV Nef stimulation was more variable within each immunization group and therefore while peak values were elevated during the boosting phase (Table 1), only the week 38 time point showed significant differences for the non-boosted and gp140 boosted groups above control ($p=0.0022$ for each).

Although less potent than Env, Gag-specific T cell proliferation was also significantly higher in comparison to controls following the second Ad priming (peak SI of 4.3, Table 1), with $p=0.0002$ at week 14, and $p=0.0004$ at week 22. In contrast to the ELISpot results, boosting with gp140 did not result in detectable proliferative responses to Gag during this period. However a single peptomer immunization did significantly boost responses to Gag at week 26 ($p=0.0022$), that were sustained until the time of challenge, but at lower levels ($p=0.03$). In fact, the peptomer boost elicited the broadest virus-specific proliferative responses, including responses to the $SHIV_{89.6P}$ peptomer itself as well as to SIV Nef, SIV Gag, AT-2 inactivated $SHIV_{89.6P}$, and $HIV_{89.6P}$ gp140 (Table 1). The peptomer

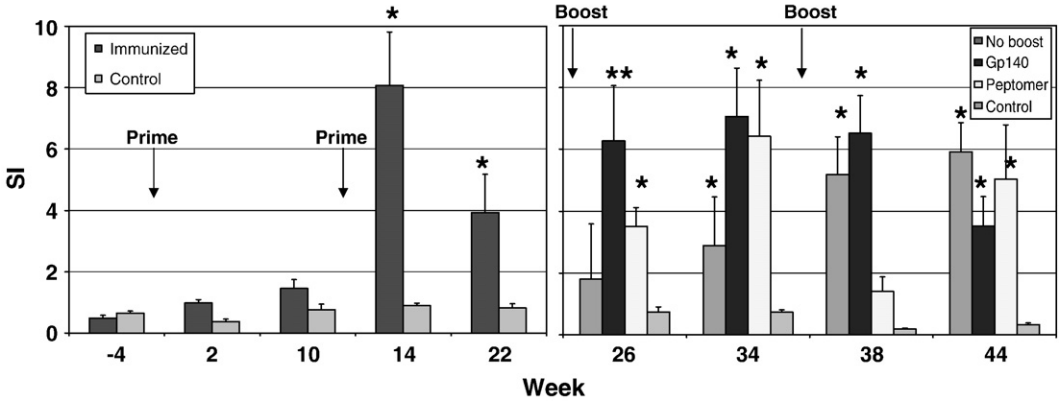

Fig. 2. T cell proliferative responses after stimulation with $HIV_{89.6P}$ Env protein. All immunized macaques received the same Ad recombinant priming vectors and so are averaged together until boosting. Group means are shown±sem. The double asterisk for the gp140 boosted group at week 26 denotes that in addition to being significantly different from the controls, it is also different from the non-boosted group.

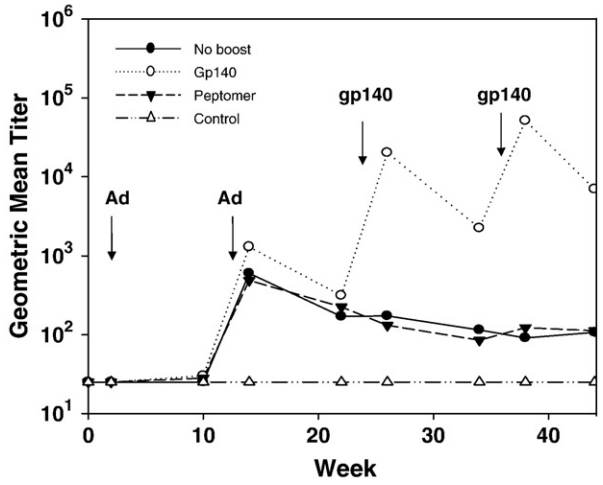

Fig. 3. Geometric mean binding antibody titers during immunization. Sera collected at multiple time points during immunization were tested for reactivity to $HIV_{89.6P}$ Env protein in an ELISA. Antibody titers were defined as the reciprocal dilution at which the absorbance measured was two times above a 1:50 dilution of the control serum.

sequence is known to contain a potent T-helper epitope which again, may stimulate responses to heterologous proteins such as Gag and/or Nef via bystander activation.

Binding antibodies are induced after the second Ad recombinant priming and boosted along with ADCC activity after gp140 immunization

Using $HIV_{89.6P}$ gp140 recombinant protein to coat ELISA plates, binding antibody levels were measured at numerous time points after immunization. Geometric mean titers are shown in Fig. 3. After the second Ad-recombinant immunization, anti-Env binding titers rose to averages of 500–1290 for the three immunized groups. The mean titer for the immunized macaques was significantly higher than that of the controls ($p=0.0004$, week 14; $p=0.0063$, week 22). Upon boosting with gp140, mean titers dramatically increased two weeks following each boost to 20,079 and 51,250 at weeks 26 and 38, respectively. At all time points titers were significantly different from controls and the non-boosted and peptomer boosted macaques ($p<0.0002$). Anti-Env titers for the non-boosted and peptomer-boosted monkeys were not significantly different from controls at any point after week 22.

ADCC activity was measured for all macaques at week 42, two weeks prior to challenge. In accordance with the binding antibody titers, only the gp140 boosted group mediated ADCC, exhibiting a geometric mean titer of 400. All other groups had titers of 10 or less (Table 2).

Neutralizing antibodies assessed by the luciferase assay on TZM-bl cells were not detectable during the immunization course against either $SHIV_{89.6P}$ pseudovirus or $SHIV_{89.6P}$ PBMC-grown virus stocks at serum dilutions of 1:20 (data not shown).

Virus-specific, intracellular cytokine responses detectable in both central and effector memory T cells in PBMC, lymph node biopsies and BAL post Ad priming and gp140 protein boosting

Given that our vaccine was delivered mucosally and that mucosal immunity is thought to be crucial to the efficacy of an HIV vaccine, we prospectively collected and systematically analyzed immune responses in multiple tissue compartments. The lung is thought to be an effector site similar to the gut (Picker et al., 2004), during SIV infection. However, few groups have looked at immune cells elicited in the lung during immunization. The lung is especially relevant here because our immunization route is intranasal and intratracheal, with the potential to elict a localized immune response in the upper respiratory tract. We were able to repeatedly obtain fresh lymphocytes from BAL, lymph nodes and blood for analysis of T cell memory responses following stimulation with the same Env, Gag and Nef peptide pools used for previous assays. Both freshly isolated PBMC and inguinal lymph node biopsies yielded sufficient numbers of lymphocytes which allowed antigen stimulation and staining for both $CD8^+$ and $CD4^+$ T cells. Using a representative PBMC sample from week 14, we show in Fig. 4 the gating strategy used for analyzing cytokine specific central and effector memory T cell responses by intracellular staining. Within the initial lymphocytic gate, the total $CD8^+$ or $CD4^+$ lymphocytes were identified. Further gating divided these into central ($CD28^+CD95^+$) or effector ($CD28^-CD95^+$) memory populations (CM or EM) (Pitcher et al., 2002). The IFN-γ-secreting central and effector memory cells within each memory gate were enumerated and are presented here as a percentage (Fig. 5). Although both $CD4^+$

Table 2
Peak virus-specific immune responses

Pre-challenge									Post-challenge		
Group	IFN-γ Elispot		Proliferation	Binding antibody	ADCC	% Env-specific memory T cells			IFN-γ Elispot	Env binding	Antibody
	Env	Gag	Env	Env	Env	PBMC	LN	BAL	Env-Wk 1	Week 1	Week 2
No boost	50±24	126±39	5.9±2.3	173	10	0.09	0.27	0.15	130±104	101	44,863
Gp140	332±96	397±75	7.1±1.6	51,250	400	1.09	1.7	2.21	414±143	6306	66,752
Peptomer	104±39	184±109	6.4±1.8	131	10	0.58	0.47	0.49	53±18	76	237,879
Control	11±21	118±65	.74±07	25	<10	0.27	0.07	0.16	14±10	25	25

Elispot reported as group mean SFC/10^6 PBMC. Proliferation reported as group mean SI±sem. Env-specific binding antibody and ADCC titers shown are geometric group means. % peak IFN-γ^+ responses for either $CD8^+$ or $CD4^+$ CM or EM T cells (see Fig. 5).

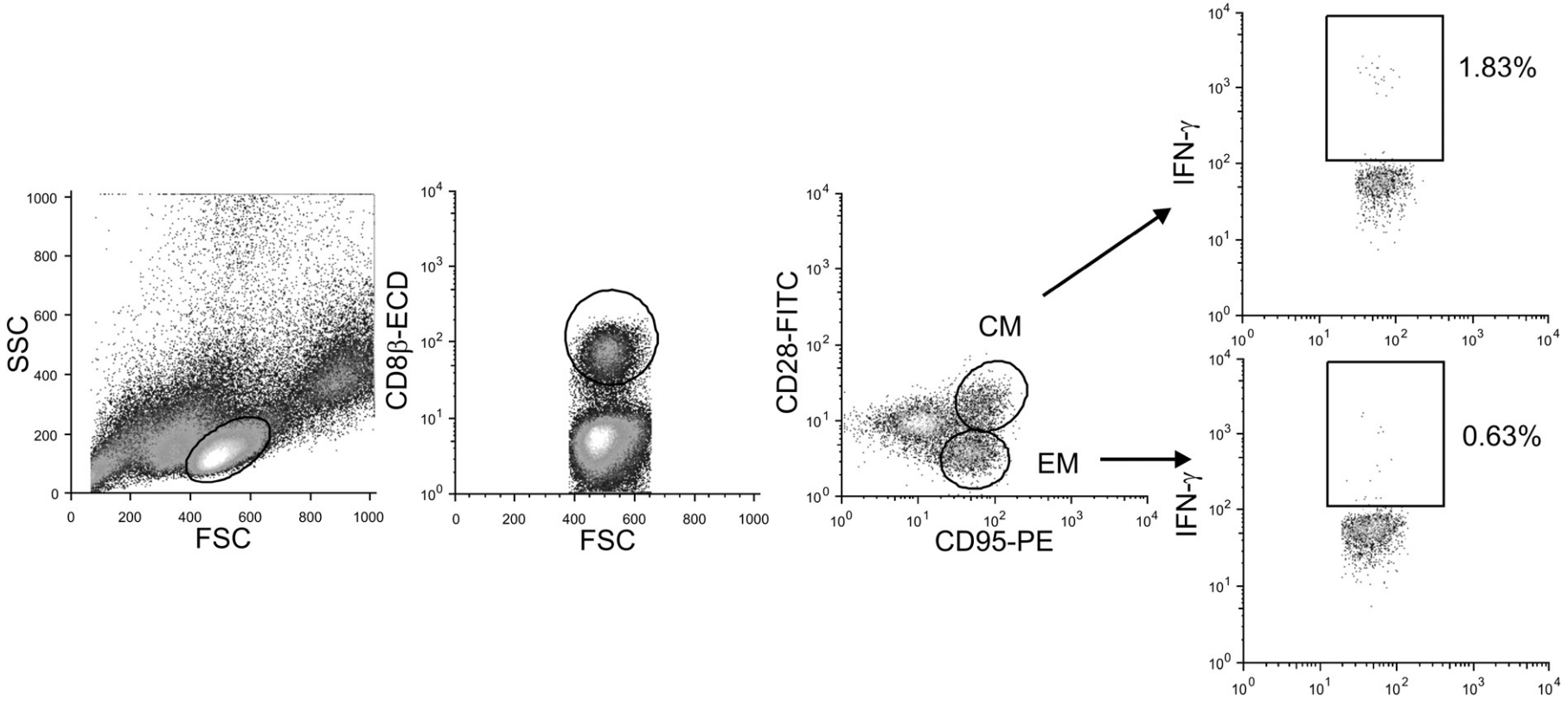

Fig. 4. Representative flow cytometric analysis of PBMC showing gating strategy used to enumerate cytokine secreting memory subsets. Shown are results after Env peptide stimulation for macaque CK6C at week 14, two weeks post second Ad recombinant priming. After gating on lymphocytes, subgating was performed first on $CD8\beta^+$ T cells (or alternatively $CD4^+$ T cells), then on central ($CD28^+CD95^+$) and effector ($CD28^-CD95^+$) memory cells. Finally, percent IFN-γ^+ cells among both the central and effector populations were determined.

and CD8$^+$ IFN-γ$^+$ cells were evident in blood and lymph nodes, only CD8 responses are shown. Mean CD4$^+$ responses were overall lower and none was significantly different from the mean of the control macaques. Only CD8$^+$ memory T cells were evaluated in BAL, as the number of cells obtained precluded measurement of both CD4$^+$ and CD8$^+$ responses.

At week 14, two weeks after the second Ad priming immunization, IFN-γ$^+$ CD8$^+$ CM and EM cells were expanded in all tissues (Fig. 5A). Central memory IFN-γ$^+$ cells were detected in peripheral blood, after stimulation with Env peptides, and to a lesser degree with Nef peptides. Although low, significant levels averaging 0.22% for Env ($p=0.0029$) and 0.13% for Nef ($p=0.045$) in blood were reached compared to the control group. Both CM and EM IFN-γ$^+$ secreting cells were observed in lymph node biopsies, however, unlike in the blood, none were significantly different from the controls.

As predicted, the number of cytokine secreting cells in response to antigenic stimulation was markedly higher in the lung. The majority of cells were EM, but CM cells were also evident, and in concert, both cell types responded to stimulation with all three antigens. Nef-specific EM responses were highest, averaging 1.63%, with 1.08% of CM responding as well. Gag CM and EM levels reached 1.3% and 0.63% respectively, while Env values were not much higher than control background levels. Even though responses were quantitatively higher than in blood, statistically they were not different from controls, due in part to the high variability among animals in these groups as evidenced by the error bars. Nevertheless, the overall trend for a

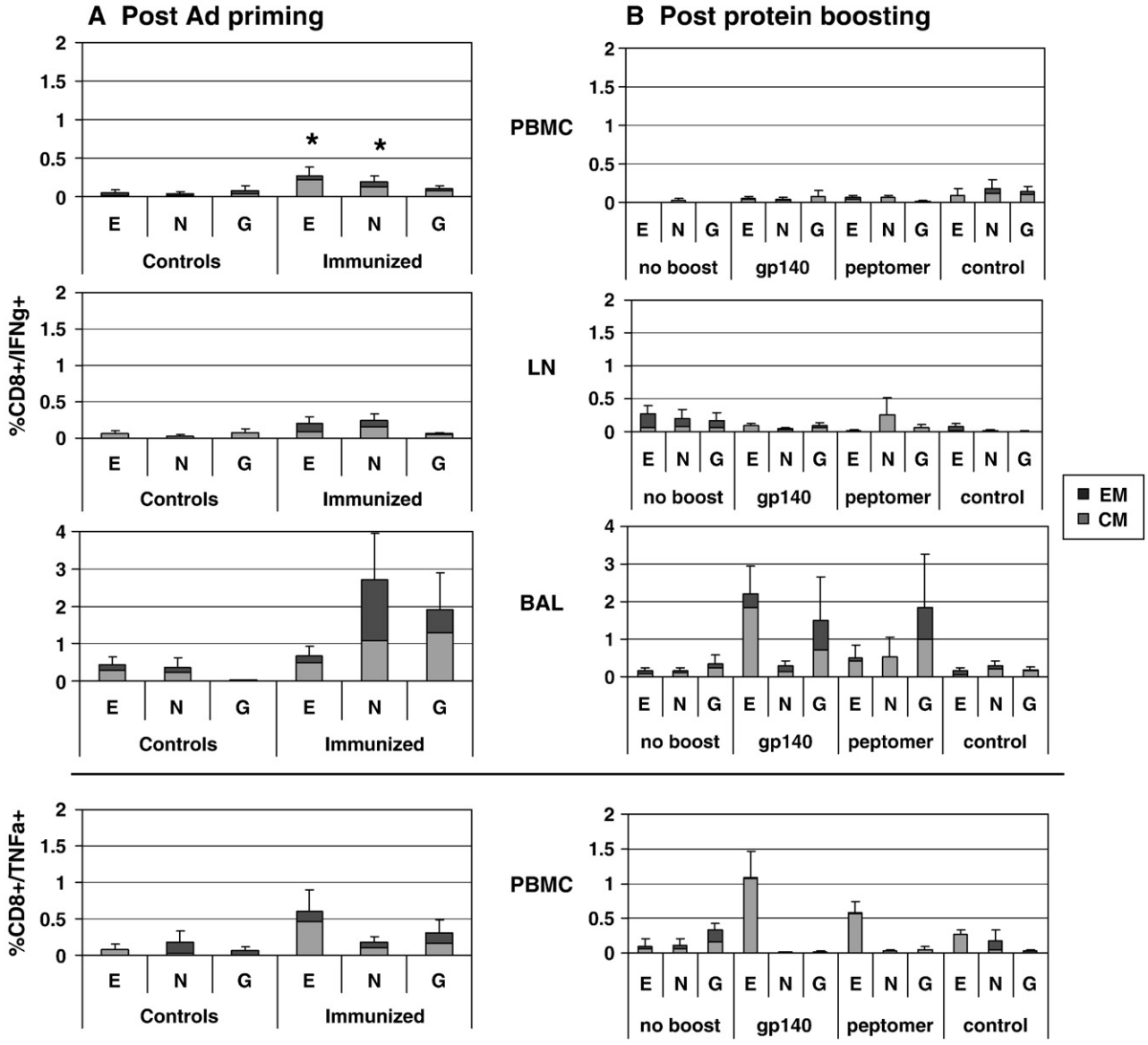

Fig. 5. IFN-γ$^+$-specific CD8$^+$ CM and EM responses in PBMC, LN and BAL after stimulation with HIV$_{89.6P}$ Env, SIV Gag, or SIV Nef peptide pools. All samples were processed and assayed immediately. After identifying the lymphocytic gate using FSC/SSC, central (CD28$^+$CD95$^+$) and effector (CD28$^-$CD95$^+$) memory cell populations were further identified as a subgate of the CD8$^+$ lymphocytes. The percentage of each memory population secreting IFN-γ or TNF-α is shown as an average for each group ± sem. As asterisk denotes that the group means are significantly different from the control group.

diverse, rapidly expanding population of memory cells was readily apparent in a typical effector site such as the lung which has also been reported elsewhere (Gauduin et al., 2006).

Finally, in addition to IFN-γ, a subset of macaques within each group was also evaluated for TNF-α-secreting memory T cells post-Ad priming at week 14 (Fig. 5A). While we did not determine whether the same cell was secreting multiple cytokines, it was clear that IFN-γ was not the sole cytokine secreted in response to antigenic stimulation, as has been shown by other groups (Wille-Reece et al., 2006).

A completely different profile was reflected in responses measured two weeks after the last protein boost, at week 38 (Fig. 5B). In peripheral blood and lymph nodes, very few if any IFN-γ^+ secreting cells were detectable, be it CM or EM.

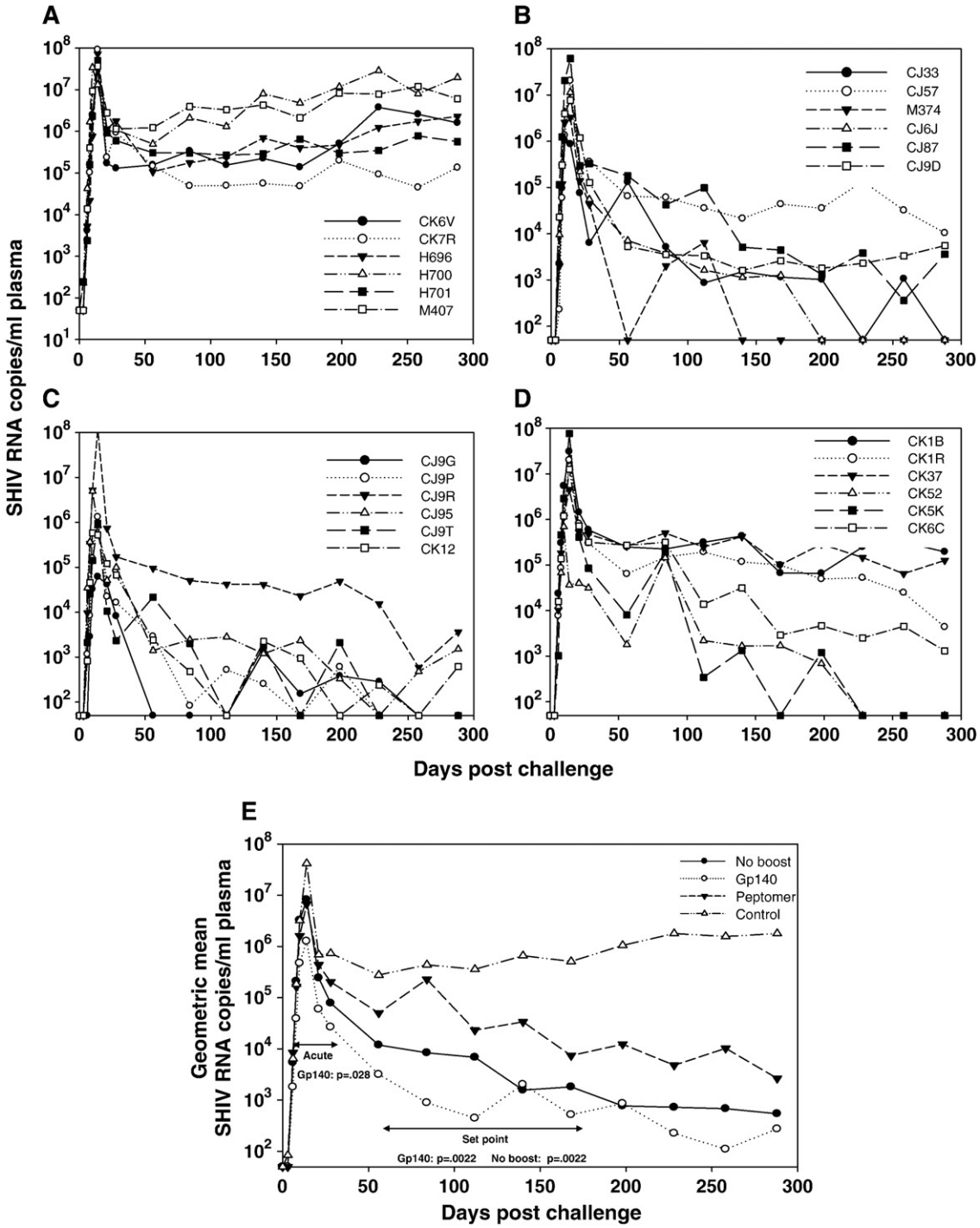

Fig. 6. Viral loads following $SHIV_{89.6P}$ challenge. Viremia levels of individual control macaques are shown in panel A, non-boosted in B, Gp140 in panel C, and peptomer boosted macaques in panel D. Geometric means for each group are shown in panel E for comparison. Statistically significant differences were found during acute phase (day 8–week 3) and set point viremia (week 8–24) for groups listed.

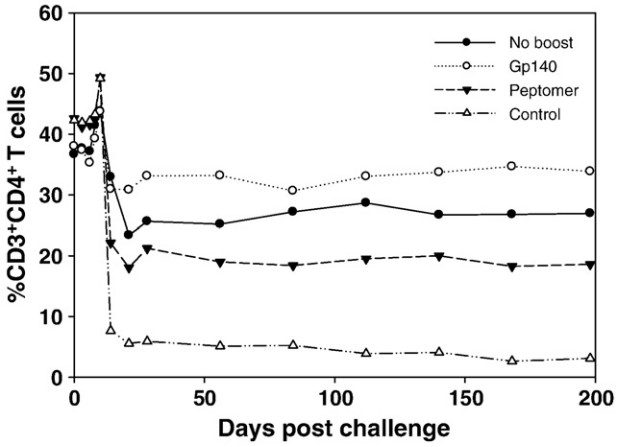

Fig. 7. %$CD3^+CD4^+$ T cells in PBMC post challenge. Geometric means for each group are shown.

However, the importance of analyzing multiple cytokines was highlighted by the observation that more than 1% of all Env-specific CM cells were TNF-α^+ in PBMC of the gp140 boosted macaques (Fig. 5B, bottom panel). In macaques boosted with gp140, proliferation of Env-specific $CD8^+$ IFN-γ^+ cells were identified in BAL, averaging 1.60% of all CM cells. This observation again points to the value of measuring immune responses at this and other effector sites when possible in order to assess the impact of vaccination and potential correlates of protection. Gag-specific responses for CM and EM were readily measured in the gp140- and peptomer-boosted monkeys as well, but were not statistically significant compared to controls.

Rectal pinch biopsies were also collected prior to immunization and following the Ad priming and protein boosting phases of the vaccine regimen in order to evaluate memory T cell responses. However, meaningful interpretation of the data was not possible since cells recovered were too few in number. Therefore, results are not reported here.

Gp140 boosting resulted in significant reduction in acute phase and set point viremia, while the non-boosted group exhibited reduced set point viremia

Results of the intravenous challenge with 90 MID_{50} of $SHIV_{89.6P}$ are shown for individual macaques (Figs. 6A–D) along with the geometric mean of each group (Fig. 6E). Significant reduction of acute phase viremia (days 8 to 21) by 1.5 logs was evident for the gp140 boosted macaques in comparison to controls ($p=0.028$ by the Wei–Johnson test), but not to the other immunized groups. Although a slight reduction in acute viremia (0.5 logs) was measured, no significant differences were observed for either the non-boosted or peptomer boosted groups versus controls. The set point was defined here as the median of the log viral loads over weeks 8–24 (days 56–168). The non-boosted and gp140 boosted macaques exhibited a significant reduction in set point viremia (roughly 3 logs) in comparison to control macaques ($p=0.0022$ for both). For the peptomer boosted group there was a reduction in viral load compared to controls over the set point phase except at week 12 (day 84), where a transient rise in viral loads was observed. This increase contributed to the lack of a significant difference between the peptomer group and the controls ($p=0.093$).

Preservation of $CD4^+$ T cells is known to be a distinct correlate of protection in the SHIV challenge model (Igarashi et al., 2002; Reimann et al., 1996); therefore the cells were monitored here following $SHIV_{89.6P}$ challenge. The reduction in percent $CD3^+$ $CD4^+$ cells post-challenge for all groups is shown in Fig. 7. There was a significant preservation of $CD3^+$ $CD4^+$ T cells for all the immunized groups compared to controls with $p<0.01$ for day 28 and thereafter. The CD4 counts were highly correlated with viral loads during both the acute and chronic phases of infection ($p=0.0077$ for days 14–56 and $p=0.0014$ for days 112–196). Due to variability within each immunization group, there were no significant differences observed between them.

Furthermore, to elucidate the basis for the significantly reduced acute viremia in the gp140-boosted group of macaques, and the overall lower viral load seen in this group during the chronic phase of infection, we investigated whether any immune responses were correlated with viremia outcomes. Although no single pre-challenge immune response in the gp140-boosted macaques was significantly correlated with either acute or chronic reductions in viral burdens, this group of animals displayed the most potent vaccine-induced immunity over the course of immunization as summarized in Table 2. The gp140-boosted macaques exhibited strong Env and Gag specific ELISpot responses, Env proliferation, and Env-specific binding antibodies that mediated ADCC activity during the boosting period and extending to the time of challenge. The greater level of responses overall in this group suggests a basis for the better challenge outcome. Responses measured in the non-boosted group were not predictive of control of chronic phase viremia. In the absence of a protein boost however, this group displayed a strong Env and Nef-specific proliferative response at the time of challenge which we speculate may have contributed to control.

With regard to post-challenge immune responses, an anamnestic IFN-γ^+ ELISpot response was seen 1 week after challenge for the gp140-boosted group (Table 2) which was significantly different from all other groups ($p=0.0022$ vs control or peptomer groups, $p=0.041$ vs no boost group). However, it was not significantly correlated with reduced peak

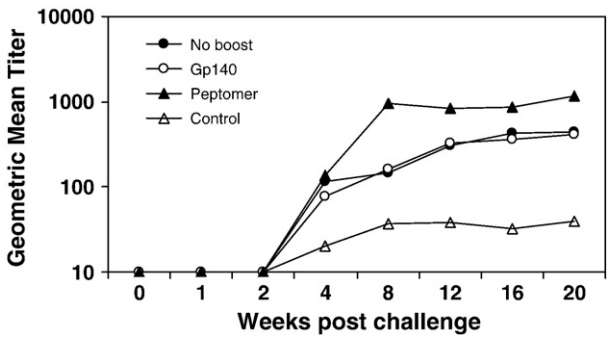

Fig. 8. Neutralizing antibody titers post challenge. Geometric mean ID_{50} neutralization titers against a $SHIV_{89.6P}$ pseudovirus using the TZM-bl Luc cell line are shown for all immunization groups post challenge.

or set point viremia. Binding antibody titers at week 1 post challenge for all immunized macaques correlated significantly with reduced viremia at week 16 post challenge ($p=0.017$). This was largely attributable to the persistent binding antibody response induced by the pre-challenge vaccine regimen exhibited by the gp140-boosted macaques (Table 2). By week 2 post-challenge, a strong anamnestic antibody response was observed for all immunized groups, whereas titers in control animals remained low (Table 2). By four weeks post-challenge, geometric mean binding titers for all immunized groups ranged from 210,000–521,000, while control titers exhibited a mean of 100 (data not shown). This outcome reflects not only the gp140 boosting, but also the priming with Ad5hr-recombinants expressing the *env* gene.

Neutralizing antibody was first observed in all the immunized macaques four weeks post-challenge with geometric mean titers ranging from 78–138 (Fig. 8). The titers gradually increased to geometric means of 420–1100 by 20 weeks post-challenge. During this same time period, control macaques exhibited a comparatively delayed and low level peak neutralizing titer of 50. The neutralizing antibody response, clearly primed by the immunization regimen, did not impact the acute phase of infection, and like the induction 2 to 4 weeks post-challenge of elevated binding antibody titers, did not necessarily predict the observed differences in chronic viremia between groups.

Discussion

Here we report results of a comparative study of the contribution of novel envelope protein boosts to immunogencity and protective efficacy in a vaccine regimen using replicating Ad5hr-HIV/SIV recombinant priming in rhesus macaques. Significant reduction in acute viremia was seen in the gp140-boosted group, signaling a clear benefit of the protein boost in the vaccine regimen. Strong Env-specific antibody and T cell responses were readily detected. Earlier results from our group using an SIV macaque model showed that binding antibodies at the time of challenge – later determined to mediate ADCC – correlated with reduction in acute phase viremia following a SIV_{mac251} challenge (Gomez-Roman et al., 2005). Here, following evaluation of ADCC activity against target cells coated with $HIV_{89.6P}$ gp140ΔCFI protein, such a correlation was not observed. However, the ADCC mediating antibody titers of 1:400 were lower compared to those that correlated with acute phase protection in the previously reported SIV challenge study (titers of 1:100,000). More extensive analyses of ADCC activity against $SHIV_{89.6P}$-infected cells pre- and post-challenge, other Fc receptor-mediated antibody activities, and mucosal antibodies are on-going in order to assess the possible contribution of envelope antibody to acute phase protection. The elevated binding antibody in the gp140-boosted animals which persisted post-challenge, together with an anamnestic SIV-specific IFN-γ ELISpot response seen one week post challenge may have contributed to the better protection observed, although taken individually a correlation was not revealed. While binding antibody responses are not generally associated with control of chronic viremia, we recently showed a combination Ad-HIV*tat/env* prime, protein boost immunization regimen elicited enhanced binding antibodies at the time of challenge that provided an explanation for greater control of chronic viremia, although a direct correlation was not observed (Demberg et al., 2007). These antibodies were non-neutralizing, but their other functional properties have yet to be examined.

Virus-specific immune responses associated with significantly reduced chronic viremia in the gp140 macaques could not be elucidated, save for the 1 week post-challenge antibody response that was correlated with reduced viremia at week 16 during the chronic phase. We speculate that the sum total of immune responses were associated with the overall better challenge outcome in the gp140 boosted animals. Further, the possible contribution of the adjuvant used in the gp140 boosting regimen to the challenge results cannot be discounted, since the other immunized macaques received only PBS or antigen diluted in PBS. However, half of the control macaques received adjuvant following priming with Ad empty-vector with no corresponding increase in non-specific immune responses detected in comparison to PBS controls. Appropriately designed studies using the identical boosting protein with or without adjuvant would address this.

The replicating Ad5hr recombinants primed for expansion of virus-specific memory $CD8^+$ T cells, both in peripheral blood and lymph nodes, although elevated numbers of cytokine producing memory $CD8^+$ T cells were seen in the lung (Fig. 5A), consistent with the intranasal/intratracheal immunization routes. These virus-specific memory cells persisted through the boosting phase at the local upper respiratory site and in peripheral blood, as shown by the TNFα-secreting cells, particularly in the gp140 and peptomer boosted groups (Fig. 5B). Memory T cells did not appear to persist in the non-boosted group, however. This response pattern does not correlate with the challenge outcome, in which the non-boosted and gp140-boosted groups showed the best protection during the chronic phase, whereas the peptomer group was not statistically different from the control macaques. This observation suggests that elements in addition to T-cell immunity, perhaps antibody responses, were contributing to protection in the gp140-boosted group. Further, the result suggests vaccine-elicited memory T-cells that contributed to protection in the non-boosted group were perhaps located in tissues other than those reported here. Alternatively, populations of vaccine-elicited memory T cells may have been present, but secreting cytokines other than IFN-γ or TNFα. In support of this notion, we observed a general two-fold expansion of total $CD8^+$ memory cells at week 38 in PBMC of both the non-boosted and gp140 boosted groups (data not shown). As both groups effectively controlled set point viremia in comparison to the peptomer and control groups, these cells may have possessed other functions that contributed to viremia control.

The fact that Ad recombinant priming alone with no intervening boost gave a significant three-log reduction of chronic viremia following challenge 32 weeks after the last immunization is remarkable and compares favorably with an earlier study also conducted in *Mamu-A*01* negative rhesus macaques (Letvin et al., 2004). In that study, Letvin et al reported that a vaccine regimen consisting of three primes with DNA vaccines

encoding SIV *gag-pol-nef*+HIV R5-*env* (mismatched to the challenge stock) and a single non-replicating Ad5 boost containing identical immunogens also resulted in a three-log reduction in chronic viremia following challenge with the same $SHIV_{89.6P}$ stock. However, the challenge was administered only 12 weeks following the last immunization, and the challenge dose was 50 MID_{50}, rather than the 90 MID_{50} used here. As also observed in the gp140 boosted group, no clear immune correlate was associated with chronic control of viremia in the non-boosted group. The observation that Env-specific proliferative responses were detectable however, gradually peaking 32 weeks after priming with no intervening boost is new, and could be an important hallmark of a replicating Ad vector. Immunization resulting in a slow expansion of virus specific memory T cells either due to a prolonged exposure to antigen or a unique mechanism of antigen presentation would be optimal.

A novel SIV peptomer boost had previously afforded strong protection from a pathogenic SIV_{mac251} challenge (Malkevitch et al., 2006; Patterson et al., 2004), leading us to hypothesize that a comparable HIV peptomer boost would protect from an appropriately matched $SHIV_{89.6P}$ challenge. Although the HIV peptomer contains both B and T cell epitopes, it was not as immunogenic as the $HIV_{89.6P}$ gp140ΔCFI protein administered in adjuvant, and subsequently failed to significantly protect rhesus macaques from challenge.

It was unexpected that no enhancement in binding or neutralizing antibody was elicited by peptomer boosting prior to challenge. However post challenge, week 2 binding antibody and week 8 neutralizing titers were 3–5-fold and 1 log higher, respectively for the peptomer boosted group compared to the gp140 and non-boosted groups. This suggests that potentially unique binding and neutralizing antibody responses were primed by peptomer immunization. Repeated immunization and/or with higher doses of HIV peptomer could possibly have achieved protective levels. The immune correlates of protection after SIV peptomer boosting responsible for prior control of the more virulent SIV_{mac251} challenge have yet to be determined. Therefore we cannot attribute the lack of protection seen here in the peptomer-boosted group to a specific immune mechanism. The HIV peptomer elicited Env-specific ELISpot responses comparable to those observed following immunization with the SIV peptomer (Patterson et al., 2003, 2004). Greater proliferative responses were induced, although the assays here were conducted on fresh cells in contrast to the earlier study where viably frozen cells were used. Neither the SIV nor HIV peptomer boosts elicited comparatively high titered envelope antibody responses. It is possible that the SIV peptomer presented a conformational epitope, not reproduced by the HIV peptomer or by test antigens in assays used to evaluate immune responses, which was associated with protection. This speculation requires further structural analysis of both the SIV and HIV peptomers.

While identical control of acute viremia was seen for both non-boosted and peptomer-boosted groups, it was puzzling that only the non-boosted group showed significant control of chronic viremia while the peptomer-boosted group did not. Given that peptomer elicited a potent T cell helper response it is worth speculating that immunization resulted in expansion of virus-specific T cells that – in the absence of protective antibody titers during acute phase to blunt viremia – could be detrimental, by giving the virus a greater number of targets, tipping the balance in favor of infection, and resulting in lesser control of long term chronic viremia.

Lastly, our mucosal vaccination route coupled with the natural transmission and targeting of Ad to mucosal inductive sites suggests that the intravenous challenge administered here might not have demonstrated the greatest potential of the vaccine regimen in terms of protective efficacy. Control of viremia – especially during the acute phase – might have been greater after a mucosally delivered challenge with a CCR5-using SHIV. Such pathogenic SHIV challenge stocks have been prepared and fully evaluated and will aid us tremendously in evaluating the efficacy of our vaccines (reviewed in Vlasak and Ruprecht, 2006).

Human clinical trials of Merck's non-replicating Ad5-HIV *gag-pol-nef* vaccine were recently stopped due to lack of efficacy. Although data are still coming to light, the suggestion that people with high anti-Ad5 titers may have been more susceptible to HIV infection is noteworthy for the entire vaccine field and is clearly relevant to our replicating Ad-recombinant approach. The trend for increased susceptibility to HIV infection was most pronounced in a subset of vaccinees which received three high dose Ad5-HIV recombinant immunizations. Detailed information concerning the immune responses elicited in this subset of people along with virological and sociological data will be necessary to validate the possible enhancement and understand the basis for the observed trial outcome. In the meantime, one can speculate that pre-existing Ad5 memory cells may have been activated following re-exposure to the Ad5-based vaccine, thereby inducing $CD4^+$ T cell activation, resulting in greater numbers of target cells for HIV infection and replication. This is a testable hypothesis, and studies to address this question are already being planned by the vaccine community.

With regard to our replicating Ad vaccine approach, there are important differences in comparison to the Merck non-replicating Ad5-HIV vaccine. Our vaccine for humans will be based in Ad type 4, which has low seroprevalance in the U.S. (Ludwig et al., 1998). Worldwide seroprevalence will need to be ascertained, however there is no reason to expect geographical differences. Further, sequential boosting is planned, first with an Ad7-based vaccine in order to circumvent any vaccine-elicited anti-Ad4 immunity, and subsequently with an envelope protein subunit in order to generate potent antibody responses. We believe cellular and humoral immune responses together will elicit greater protective efficacy. Finally, we have previously shown in non-human primates that replicating Ad-recombinants elicit more potent anti-HIV cellular immunity and better prime antibody responses compared to non-replicating Ad recombinants (Peng et al., 2005), suggesting that enhanced immunity will also be generated by the replicating vaccine in people. While plans for a phase I trial of the replication-competent Ad4 vaccine approach are underway, the human studies will move forward cautiously until the vaccine community learns whether the observed trends in the Merck trial are statistically significant and gains a full understanding of the mechanisms underlying the observed results.

In summary, replication-competent Ad-recombinants – even when administered alone – elicit long-lasting immunity, providing strong reduction in chronic viremia following challenge up to 32 weeks after immunization. Further, the benefit of a protein boost coupled with our Ad-recombinant priming scheme is clearly shown in this study. Others have shown a similar benefit following priming with DNA or other non-replicating vectors (Cristillo et al., 2006; Shu et al., 2007). The envelope boost contributed to reductions in both acute phase and chronic viremia. It not only elicited humoral immunity, but in combination with replicating Ad recombinant priming additionally boosted cellular immunity. The underlying mechanism for this expansion has not been elucidated, however routine exploitation of such a vaccine-induced response would be worth incorporating into future strategies for HIV vaccine development. Further improvement in the design of the envelope protein used for boosting as well as characterization of immune responses induced by priming alone should only increase the potential of the Ad-recombinant prime/protein boost approach.

Materials and methods

Immunization and challenge schedule

Twenty-four juvenile male Indian rhesus macaques, negative for SIV, simian retrovirus type D, and simian T-cell leukemia virus, were housed according to NIH animal care guidelines at Bioqual, Inc., Rockville, Maryland. All animals were *Mamu-A*01* negative. One macaque, CJ9T, tested positive for the *B*17* allele which is overrepresented in long-term non-progressor macaques (Yant et al., 2006) but not sufficient to predict SIV disease outcome (Wojcechowskyj et al., 2007).

The immunization scheme is shown in Fig. 9. The twenty-four macaques were divided equally into four groups. Groups I, II, and III received identical replication-competent Ad5hr-HIV/SIV recombinant priming immunizations encoding $HIV_{89.6P}$ gp140ΔCFI (T. Demberg et al., submitted), SIV*gag*$_{239}$ (Zhao et al., 2003b), and SIV*nef*$_{239\Delta1-14}$ (Patterson et al., 2003) at weeks 0 (intranasal and oral routes) and 12 (intratracheally) at a dose of 5×10^8 pfu/recombinant for a total of 1.5×10^9 pfu/macaque/route of immunization. Procedures for the intranasal (0.25 ml per nostril), oral (0.5 ml via stomach tube), and intratracheal (0.5 ml) administrations have been described previously (Zhao et al., 2003a). A plasmid containing the $HIV_{89.6P}$*gp140ΔCFI* gene has been described (Letvin et al., 2004) and was generously provided by Dr. Gary Nabel, VRC, NIH, for construction of the Ad5hr-recombinant and preparation of expressed protein as described elsewhere (T. Demberg et al., submitted). Groups were distinguished by boosting immunogens administered intramuscularly at weeks 24 and 36 at a dose of 100 μg/macaque. Group I received no boost, only PBS, and Group II, $HIV_{89.6P}$ gp140ΔCFI protein in 50 μg monophosphoryl lipid A-stable emulsion (MPL-SE) adjuvant (Corixa) at a final 1:10 dilution. Group III received a $HIV_{89.6P}$ peptomer composed of repeating 18-mers covalently linked end-to-end, representing amino acids 419–436 of the envelope and formulated in PBS (Robey et al., 1995). Group IV controls received a comparable dose of empty replicating Ad5hrΔE3 vector (no insert) with 3 macaques getting MPL-SE and 3 receiving PBS during the boosting phase. All macaques were challenged intravenously at week 44 with 90 MID_{50} of a $SHIV_{89.6P}$ challenge stock obtained from Nancy Miller, Division of AIDS, NIAID, NIH, and originally prepared and titered by Keith Reimann and Norman Letvin, Beth Israel Deaconess Medical Center, Harvard Medical School.

Tissue collection and processing

Peripheral blood samples were collected at serial time points throughout the study. Lymphocytes were isolated by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, USA), and used either fresh or were frozen in fetal bovine serum (FBS, Invitrogen) containing 7% DMSO, and stored in liquid nitrogen until use. In addition, at weeks 0, 14 and 38 post immunization and weeks 2 and 12 post challenge, inguinal lymph node biopsies and bronchio-alveolar lavage (BAL) samples were processed and directly used in assays. Lymph nodes biopsies were sliced finely with a scalpel, and pressed through a 70 μm filter in order to isolate lymphocytes. For BAL, 30–50 ml of fluid was collected after deep flushing of one

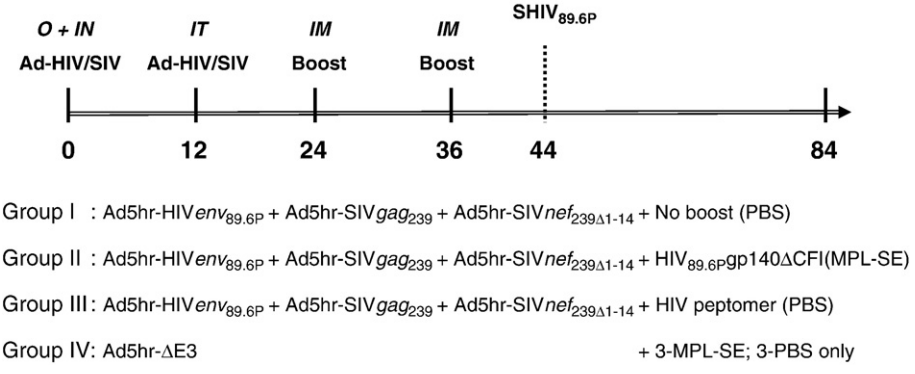

Fig. 9. Immunization regimen and challenge schedule. As detailed in Materials and methods, four groups of six *Mamu-A*01* negative macaques were primed at weeks 0 and 12 with three Ad recombinant vectors each encoding $HIV_{89.6P}$ *gp140ΔCFI*, SIV*gag*$_{239}$ or SIV*nef*$_{239\Delta1-14}$. Groups differed by boosting immunogen, receiving no boost, soluble $HIV_{89.6P}$*gp140ΔCFI* protein or an $HIV_{89.6P}$ peptomer. A control group received empty Ad vector followed with MPL-SE adjuvant or PBS only to match the boosting groups. Eight weeks after the last boost, all macaques were challenged intravenously with 90 MID_{50} of a $SHIV_{89.6P}$ stock.

bronchus with PBS. The cells were pelleted, and a small aliquot of supernatant frozen. The cell pellet was resuspended in 10 ml of PBS, then layered onto a Percoll (Sigma, St. Louis, Missouri) gradient consisting of 2 ml of 65% Percoll diluted in RPMI-1640, followed by 2 ml of 35%. After centrifugation for 30 min at 400 ×*g*, the whitish live cell layer was collected at the interface, the lymphocytes counted and resuspended in 3 ml of R10 (RPMI-1640 plus 10% heat inactivated fetal bovine serum, 1 mM L-glutamine and 100 U/ml Pen-Strep; Invitrogen). After overnight incubation, the cells were used immediately in immune assays. In addition to the above tissues, rectal pinch biopsies were also collected during this study. Unfortunately low cell numbers after processing precluded their inclusion in further data analyses.

Measurement of cellular immunity

IFN-γ ELISpot

Fresh PBMC were counted and plated in triplicate into a 96-well plate at a density of 10^5 and 5×10^4 cells/well in 100 μl R10 media. Overlapping peptides representing HIV and SIV genes were added at a concentration of 1 μg/ml of each peptide and the plate incubated overnight at 37 °C. Peptide pools consisting of 15-mers overlapping by 11 amino acids for SIV_{239} Gag and SIV_{251} Nef, and 20-mers overlapping by 10 for $HIV_{89.6P}$ Env were obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH. Control wells containing media alone or media alone plus the appropriate DMSO concentration were included, as well as a positive ConA (Sigma) control at 5 μg/ml. The next day, the plates were transferred to a 96-well plate that had been previously coated with anti-IFN-γ monoclonal antibody MD-1, and blocked with 2% BSA (200 μl/well) for 1 h at 37 °C. After a 5 h incubation at 37 °C, the plates were developed according to kit instructions (U-Cytech, Utrecht, The Netherlands). Spots were averaged, and the average of background spots was subtracted to obtain the net number of spots reported. Total spot forming cells (SFC) are shown/10^6 PBMC. On rare occasions, data were eliminated from analysis when the negative control background wells exceeded 125 SFC/10^6 PBMC.

Proliferation

Fresh PBMC were used to measure SIV_{251} Gag-, SIV_{251} Nef-, and $SHIV_{89.6P}$ gp140 Env-specific lymphoproliferative responses at each time point tested. Purified proteins which matched HIV/SIV inserts were used for stimulation. Additionally, AT-2 inactivated $SHIV_{89.6P}$ supplied by Dr. Jeff Lifson and Julian Bess (NCI-Frederick, Frederick, MD) was used along with an equal amount of empty SUPT1 microvesicles, which served as a negative control. A total of 3×10^5 cells/well plus 4 μg protein/well were plated in triplicate in a 96-well plate. On the fourth day of incubation, 1 μCi of ^{3}H-thymidine was added to each well and the plate harvested onto a Perkin Elmer filter mat the next day for determination of thymidine incorporation using a Perkin Elmer Microbeta Trilux beta counter. The stimulation index (SI) was calculated as the mean counts per minute for the stimulated well divided by the mean counts for the media only wells.

Intracellular cytokine staining

At weeks 0, 14 and 38 pre-challenge and weeks 2 and 12 post-challenge freshly isolated PBMC, lymph node and BAL samples were analyzed by intracellular cytokine staining for IFN-γ secreting $CD8^+$ and $CD4^+$ memory T cells in response to stimulation with the same $SHIV_{89.6P}$ Env, SIV_{239} Gag and SIV_{251} Nef peptides used for the ELISpot assay (see above). Fresh lymphocytes were counted and adjusted with R10 to $1–2\times10^6$ cells/ml for each stimulation condition, including a no-peptide control and a staphyloccal entertoxin B (SEB) positive control (0.2 μg/ml). After a 1 h incubation at 37 °C, 2 μl/ml of GolgiStop (BD Biosciences) was added and the tubes incubated an additional 5 h after which they were washed and stained according to previously published protocols (Demberg et al., 2007) using permeabilization/fixation solutions A and B from Invitrogen and antibodies from BD Pharmingen unless specified otherwise. Briefly, after washing twice with PBS, cells were surface-stained for 20 min using three separate antibodies including CD28-FITC (Clone 28.2), CD95-PE (Clone DX2), CD8β-ECD (Clone 2ST8.5H7, Beckman Coulter) or CD4-PerCP (Clone L200). After incubating with solution A for 15 min with washing before and after, the cells were incubated with solution B containing anti-IFNγ-APC (Clone B27) for 20 min, washed, pelleted and fixed with 3.7% formaldehyde diluted in PBS. At least 100,000 events were collected within the lymphocytic gate using a FACScalibur and Cellquest software. A positive response was defined as one significant over unstimulated cells at the two-tailed $\alpha=0.05$ level by the continuity adjusted chi-squared test. Due to the loss of power for detection, a response comparison was excluded if the harmonic mean of the gated central or effector memory event numbers was less than 300.

Measurement of humoral immunity

Serum binding antibodies reactive with $SHIV_{89.6P}$ gp140 Env protein were measured in an ELISA assay as previously described (Buge et al., 1997). The reciprocal of the dilution at which the absorbance was two times greater than a 1:50 dilution of the control serum was defined as the antibody titer.

ADCC activity of macaque serum antibodies was assessed using the rapid fluorometric ADCC assay (RFADCC) described elsewhere (Gomez-Roman et al., 2006a,b). Briefly, CEM-NK^r target cells (AIDS Research and Reference Reagent Program, National Institutes of Allergy and Infectious Diseases) were coated with $HIV_{89.6P}$ gp140 and double stained with the membrane dye, PKH-26 (Sigma Aldrich) and the vital dye, CFSE (Molecular Probes). Labeled target cells were resuspended in RPMI 1640 medium containing 10% FCS (R10) and allowed to react with heat inactivated (56 °C, 30 min), serially diluted macaque sera in a 96-well microtiter plate for 15 min at room temperature. Human effector cells were added at a E:T ratio of 50:1. The reaction mixture was incubated at 37 °C in 5% CO_2 for 4 h and fixed with 3.7% paraformaldehyde for flow cytometry acquisition (FACSCalibur instrument, Becton Dickinson, San Jose, CA, USA). Fifty thousand non-gated events from duplicate wells were acquired within 18 h using CellQuest software and data analysis was performed using WinMDI 2.9.

ADCC cell killing was calculated as the percentage of membrane-labeled target cells that lost the viability dye, i.e.: percentage of CFSE ^{negative} within the PKH-26 ^{high} gate. ADCC titer is defined as the reciprocal serum dilution at which the percent ADCC killing was greater than the mean percent killing of negative control sera plus 3 SDs.

ID_{50} neutralizing antibody titers were determined using the TZM-bl Luc cell line and either a $SHIV_{89.6P}$ pseudovirus or $SHIV_{89.6}$ PBMC-grown stock as already described (Demberg et al., 2007; Li et al., 2005a). Titers are defined as the reciprocal plasma dilution at which there was a 50% reduction in relative luminescence units (RLUs) compared to virus control wells which contained no test sample.

Measurement of viral RNA

A description of the nucleic acid sequence-based amplification technique (NASBA) used in this study was detailed in previous publications (Malkevitch et al., 2006; Romano et al., 2000). All samples were initially screened using this ECL-based assay which has a sensitivity of <2000 viral RNA copies per input plasma volume (generally 100 μl). When lower sensitivity was required, a real-time NASBA assay with a sensitivity of <50 copies/ml was utilized, thereby defining the sensitivity of detection as 50 copies.

Statistical analyses

Comparisons between groups at individual times were assessed using the exact Wilcoxon rank sum test, and comparisons over intervals used repeated measures analysis of variance for outcomes consistent with normal distributions and the Wei–Johnson method of other outcomes. For changes between times, the Wilcoxon signed rank test was applied to differences. ELISpot response data were tested after a symmetrizing power transformation (Box–Cox lambda = 0.33) that yielded normal residual distributions. Multiple pairwise tests among the three immunized groups have been corrected by the method of Hochberg.

Acknowledgments

We wish to acknowledge Dr. Marisa St. Claire and the superb technical expertise of the animal care staff at Bioqual, Inc., in particular Steve Harbaugh and Jeff Harbaugh who developed the BAL sampling procedure. Reagents were kindly provided by: Dr Gary Nabel, VRC, NIAID, NIH ($SHIV_{89.6P}$*gp140ΔCFI* gene), Dr. Jeffrey Lifson and Julian Bess (AT-2 inactivated $SHIV_{89.6P}$), and Drs. Keith Reimann and Norman L. Letvin ($SHIV_{89.6P}$ challenge stock). The following reagents were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-NK^r cells from Dr. Peter Cresswell; $SHIV_{89.6P}$ Env peptides, complete set; SIV Gag peptides, complete set, and SIV Nef peptides, complete set. This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, and NIH grant AI 30034.

References

Amara, R.R., Villinger, F., Altman, J.D., Lydy, S.L., O'Neil, S.P., Staprans, S.I., Montefiori, D.C., Xu, Y., Herndon, J.G., Wyatt, L.S., Candido, M.A., Kozyr, N.L., Earl, P.L., Smith, M.J., Ma, H.-L., Grimm, B.D., Hulsey, M.L., Miller, J., McClure, H.M., McNicholl, J.M., Moss, B., Robinson, H.L., 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 292, 69–74.

Buge, S.L., Richardson, E., Alipanah, S., Markham, P., Cheng, S., Kalyan, N., Miller, C.J., Lubeck, M., Udem, S., Eldridge, J., Robert-Guroff, M., 1997. An adenovirus-simian immunodeficiency virus env vaccine elicits humoral, cellular, and mucosal immune responses in rhesus macaques and decreases viral burden following vaginal challenge. J. Virol. 71, 8531–8541.

Catanzaro, A.T., Koup, R.A., Roederer, M., Bailer, R.T., Enama, M.E., Moodie, Z., Gu, L., Martin, J.E., Novik, L., Chakrabarti, B.K., Butman, B.T., Gall, J.G.D., King, C.R., Andrews, C.A., Sheets, R., Gomez, P.L., Mascola, J.R., Nabel, G.J., Graham, B.S., the VRC 006 Study Team, 2006. Phase I safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. J. Infect. Dis. 194, 1638–1649.

Cristillo, A.D., Wang, S., Caskey, M.S., Unangst, T., Hocker, L., He, L., Hudacik, L., Whitney, S., Keen, T., Chou, T.W., Shen, S., Joshi, S., Kalyanaraman, V.S., Nair, B., Markham, P., Lu, S., Pal, R., 2006. Preclinical evaluation of cellular immune responses elicited by a polyvalent DNA prime/protein boost HIV-1 vaccine. Virology 346, 151–168.

Demberg, T., Florese, R., Heath, M.J., Larsen, K., Kalisz, I., Kalyanaraman, V.S., Lee, E.-M., Pal, R., Venzon, D., Grant, R., Patterson, L.J., Korioth-Schmitz, B., Buzby, A., Dombagoda, D., Montefiori, D.C., Letvin, N.L., Cafaro, A., Ensoli, B., Robert-Guroff, M., 2007. A replication-competent Ad-HIV*tat* and -HIV*env* priming/Tat and Envelope protein boosting regimen elicits enhanced protective efficacy against $SHIV_{89.6P}$ challenge in rhesus macaques. J. Virol. 81, 3414–3427.

Duerr, A., Wasserheit, J.N., Corey, L., 2006. HIV vaccines: new frontiers in vaccine development. Clin. Infect. Dis. 43, 500–511.

Gauduin, M.-C., Yu, Y., Barabasz, A., Carville, A., Piatak, M., Lifson, J.D., Desrosiers, R.C., Johnson, R.P., 2006. Induction of a virus-specific effector-memory $CD4^+$ T cell response by attenuated SIV infection. J. Exp. Med. 203, 2661–2672.

Gomez-Roman, V.R., Patterson, L.J., Venzon, D., Liewehr, D., Aldrich, K., Florese, R., Robert-Guroff, M., 2005. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. J. Immunol. 174, 2185–2189.

Gomez-Roman, V.R., Florese, R.H., Patterson, L.J., Peng, B., Venzon, D., Aldrich, K., Robert-Guroff, M., 2006a. A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. J. Immunol. Methods 308, 53–67.

Gomez-Roman, V.R., Florese, R.H., Peng, B., Montefiori, D.C., Kalyanaraman, V.S., Venzon, D., Srivastava, I., Barnett, S.W., Robert-Guroff, M., 2006b. An adenovirus-based HIV subtype B prime/boost vaccine regimen elicits antibodies mediating broad antibody-dependent cellular cytotoxicity against non-subtype B HIV strains. J. Acquir. Immune Defic. Syndr. 43, 270–277.

Igarashi, T., Brown, C.R., Byrum, R.A., Nishimura, Y., Endo, Y., Plishka, R.J., Buckler, C., Buckler-White, A., Miller, G., Hirsch, V.M., Martin, M.A., 2002. Rapid and irreversible $CD4^+$ T-cell depletion induced by the highly pathogenic simian/human immunodeficiency virus $SHIV_{DH12R}$ is systemic and synchronous. J. Virol. 76, 379–391.

Letvin, N.L., Huang, Y., Chakrabarti, B.K., Xu, L., Seaman, M.S., Beaudry, K., Korioth-Schmitz, B., Yu, F., Rohne, D., Martin, K.L., Miura, A., Kong, W.P., Yang, Z.Y., Gelman, R.S., Golubeva, O.G., Montefiori, D.C., Mascola, J.R., Nabel, G.J., 2004. Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. J. Virol. 78, 7490–7497.

Li, M., Gao, F., Mascola, J.R., Stamatatos, L., Polonis, V.R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K.M., Bilska, M., Kothe, D.L., Salazar-Gonzalez, J.F., Wei, X., Decker, J.M., Hahn, B.H., Montefiori, D.C., 2005a. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. 79, 10108–10125.

Li, Q., Duan, L., Estes, J.D., Ma, Z.M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C.J., Haase, A.T., 2005b. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. Nature 434, 1148–1152.

Liang, X., Casimiro, D.R., Schleif, W.A., Wang, F., Davies, M.E., Zhang, Z.Q., Fu, T.M., Finnefrock, A.C., Handt, L., Citron, M.P., Heidecker, G., Tang, A., Chen, M., Wilson, K.A., Gabryelski, L., McElhaugh, M., Carella, A., Moyer, C., Huang, L., Vitelli, S., Patel, D., Lin, J., Emini, E.A., Shiver, J.W., 2005. Vectored Gag and Env but not Tat show efficacy against simian–human immunodeficiency virus 89.6P challenge in *Mamu-A*01*-negative rhesus monkeys. J. Virol. 79, 12321–12331.

Lubeck, M.D., Natuk, R.J., Myagkikh, M., Kalyan, N., Aldrich, K., Sinangil, F., Alipanah, S., Murthy, S.C.S., Chanda, P.K., Nigida, S., Markham, P.D., Zolla-Pazner, S., Steimer, K., Wade, M., Reitz Jr., M.S., Arthur, L.O., Mizutani, S., Davis, A., Hung, P., Gallo, R.D., Eichberg, J., Robert-Guroff, M., 1997. Long-term protection of chimpanzees against high-dose HIV-1 challenge introduced by immunization. Nat. Med. 3, 651–658.

Ludwig, S.L., Brundage, J.F., Kelley, P.W., Nang, R., Towle, C., Schnurr, D.P., Crawford-Miksza, L., Gaydos, J.C., 1998. Prevalence of antibodies to adenovirus serotypes 4 and 7 among unimmunized US Army trainees: results of a retrospective nationwide seroprevalence survey. J. Infect. Dis. 178, 1776–1778.

Malkevitch, N.V., Patterson, L.J., Aldrich, M.K., Wu, Y., Venzon, D., Florese, R.H., Kalyanaraman, V.S., Pal, R., Lee, E.M., Zhao, J., Cristillo, A., Robert-Guroff, M., 2006. Durable protection of rhesus macaques immunized with a replicating adenovirus-SIV multigene prime/protein boost vaccine regimen against a second SIV(mac251) rectal challenge: Role of SIV-specific CD8+ T cell responses. Virology 353, 83–98.

Mascola, J.R., Sambor, A., Beaudry, K., Santra, S., Welcher, B., Louder, M.K., Vancott, T.C., Huang, Y., Chakrabarti, B.K., Kong, W.P., Yang, Z.Y., Xu, L., Montefiori, D.C., Nabel, G.J., Letvin, N.L., 2005. Neutralizing antibodies elicited by immunization of monkeys with DNA plasmids and recombinant adenoviral vectors expressing human immunodeficiency virus type 1 proteins. J. Virol. 79, 771–779.

Mattapallil, J.J., Douek, D.C., Hill, B., Nishimura, Y., Martin, M., Roederer, M., 2005. Massive infection and loss of memory $CD4^+$ T cells in multiple tissues during acute SIV infection. Nature 434, 1093–1097.

Mothe, B.R., Weinfurter, J., Wang, C., Rehrauer, W., Wilson, N., Allen, T.M., Allison, D.B., Watkins, D.I., 2003. Expression of the major histocompatibility complex class I molecule *Mamu-A*01* is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 77, 2736–2740.

Patterson, L.J., Malkevitch, N., Pinczewski, J., Venzon, D., Lou, Y., Peng, B., Munch, C., Leonard, M., Richardson, E., Aldrich, K., Kalyanaraman, V.S., Pavlakis, G.N., Robert-Guroff, M., 2003. Potent, persistent induction and modulation of cellular immune responses in rhesus macaques primed with Ad5hr-simian immunodeficiency virus (SIV) env/rev, gag, and/or nef vaccines and boosted with SIV gp120. J. Virol. 77, 8607–8620.

Patterson, L.J., Malkevitch, N., Venzon, D., Pinczewski, J., Gomez-Roman, V.R., Wang, L., Kalyanaraman, V.S., Markham, P.D., Robey, F.A., Robert-Guroff, M., 2004. Protection against mucosal simian immunodeficiency virus SIV (mac251) challenge by using replicating adenovirus-SIV multigene vaccine priming and subunit boosting. J. Virol. 78, 2212–2221.

Peng, B., Wang, L.R., Gomez-Roman, V.R., Davis-Warren, A., Montefiori, D.C., Kalyanaraman, V.S., Venzon, D., Zhao, J., Kan, E., Rowell, T.J., Murthy, K.K., Srivastava, I., Barnett, S.W., Robert-Guroff, M., 2005. Replicating rather than nonreplicating adenovirus-human immunodeficiency virus recombinant vaccines are better at eliciting potent cellular immunity and priming high-titer antibodies. J. Virol. 79, 10200–10209.

Picker, L.J., Hagen, S.I., Lum, R., Reed-Inderbitzin, E.F., Daly, L.M., Sylwester, A.W., Walker, J.M., Siess, D.C., Piatak Jr., M., Wang, C., Allison, D.B., Maino, V.C., Lifson, J.D., Kodama, T., Axthelm, M.K., 2004. Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. J. Exp. Med. 200, 1299–1314.

Pitcher, C.J., Hagen, S.I., Walker, J.M., Lum, R., Mitchell, B.L., Maino, V.C., Axthelm, M.K., Picker, L.J., 2002. Development and homeostasis of T cell memory in rhesus macaque. J. Immunol. 168, 29–43.

Reimann, K.A., Li, J.T., Veazey, R., Halloran, M., Park, I.-W., Karlsson, G.B., Sodroski, J., Letvin, N.L., 1996. A chimeric simian/human immunodeficiency virus type 1 isolate *env* causes an AIDS-like disease after in vivo passage in rhesus macaques. J. Virol. 70, 6922–6928.

Robert-Guroff, M., Kaur, H., Patterson, L.J., Leno, M., Conley, A.J., McKenna, P.M., Markham, P.D., Richardson, E., Aldrich, K., Arora, K., Murty, L., Carter, L., Zolla-Pazner, S., Singangil, F., 1998. Vaccine protection against a heterologous, non-syncytium-inducing, primary human immunodeficiency virus. J. Virol. 72, 10275–10280.

Robey, F.A., Kelson-Harris, T., Roller, P.P., Robert-Guroff, M., 1995. A helical epitope in the C4 domain of HIV glycoprotein 120. J. Biol. Chem. 270, 23918–23921.

Robey, F.A., Harris-Kelson, T., Robert-Guroff, M., Batinic, D., Ivanov, B., Lewis, M., Roller, P.P., 1996. A synthetic conformational epitope from the C4 domain of HIV gp120 that binds CD4. J. Biol. Chem. 271, 17990–17995.

Romano, J.W., Shurtliff, R.N., Dobratz, E., Gibson, A., Hickman, K., Markham, P.D., Pal, R., 2000. Quantitative evaluation of simian immunodeficiency virus infection using NASBA technology. J. Virol. Methods 86, 61–70.

Rose, N.F., Marx, P.A., Luckay, A., Nixon, D.F., Moretto, W.J., Donahoe, S.M., Montefiori, D.C., Roberts, A., Buonocore, L., Rose, J.K., 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell 106, 539–549.

Shiver, J.W., Fu, T.-M., Chen, L., Casimiro, D.R., Davies, M.E., Evans, R.K., Zhang, Z.-Q., Simon, A.J., Trigona, W.L., Dubey, S.A., Huang, L., Harris, V.A., Long, R.S., Liang, X., Handt, L., Schleif, W.A., Zhu, L., Freed, D.C., Persaud, N.V., Guan, L., Punt, K.S., Tang, A., Chen, M., Wilson, K.A., Collins, K.B., Heidecker, G.J., Fernandez, V.R., Perry, H.C., Joyce, J.G., Grimm, K.M., Cook, J.C., Keller, P.M., Kresock, D.S., Mach, H., Troutman, R.D., Isopi, L.A., Williams, D.M., Xu, Z., Bohannon, K.E., Volkin, D.B., Montefiori, D.C., Miura, A., Krivulka, G.R., Lifton, M.A., Kuroda, M.J., Schmitz, J.E., Letvin, N.L., Caulfield, M.J., Bett, J.A., Youil, R., Kaslow, D.C., Emini, E.A., 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 415, 331–335.

Shu, Y., Winfrey, S., Yang, Z., Xu, L., Rao, S.S., Srivastava, I., Barnett, S.W., Nabel, G.J., Mascola, J.R., 2007. Efficient protein boosting after plasmid DNA or recombinant adenovirus immunization with HIV-1 vaccine constructs. Vaccine 25, 1398–1408.

Veazey, R.S., DeMaria, M., Chalifoux, L.V., Shvetz, D.E., Pauley, D.R., Knight, H.L., Rosenzweig, M., Johnson, R.P., Desrosiers, R.C., Lackner, A.A., 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science 280, 427–431.

Veazey, R.S., Tham, I.C., Mansfield, K.G., DeMaria, M., Forand, A.E., Shvetz, D.E., Chalifoux, L.V., Sehgal, P.K., Lackner, A.A., 2000. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection *in vivo*. J. Virol. 74, 57–64.

Vlasak, J., Ruprecht, R.M., 2006. AIDS vaccine development and challenge viruses: getting real. AIDS 20, 2135–2140.

Wille-Reece, U., Flynn, B.J., Lore, K., Koup, R.A., Miles, A.P., Saul, A., Kedl, R.M., Mattapallil, J.J., Weiss, W.R., Roederer, M., Seder, R.A., 2006. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. J. Exp. Med. 203, 1249–1258.

Wojcechowskyj, J.A., Yant, L.J., Wiseman, R.W., O'Connor, S.L., O'Connor, D.H., 2007. Control of simian immunodeficiency virus SIVmac239 is not predicted by inheritance of *Mamu-B*17*-containing haplotypes. J. Virol. 81, 406–410.

Yant, L.J., Friedrich, T.C., Johnson, R.C., May, G.E., Maness, N.J., Enz, A.M., Lifson, J.D., O'Connor, D.H., Carrington, M., Watkins, D.I., 2006. The high-frequency major histocompatibility complex class I allele *Mamu-B*17* is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 80, 5074–5077.

Zhang, Z.-Q., Fu, T.-M., Casimiro, D.R., Davies, M.E., Liang, X., Schleif, W.A., Freed, D.C., Tan, C.Y., Horton, M., Emini, E.A., Shiver, J.W., 2002. *Mamu-A*01* allele-mediated attenuation of disease progression in simian human immunodeficiency virus infection. J. Virol. 76, 12845–12854.

Zhao, J., Pinczewski, J., Gomez-Roman, V.R., Venzon, D., Kalyanaraman, V.S., Markham, P.D., Aldrich, K., Moake, M., Montefiori, D.C., Lou, Y., Pavlakis, G.N., Robert-Guroff, M., 2003a. Improved protection of rhesus macaques against intrarectal simian immunodeficiency virus SIV_{mac251} challenge by a replication-competent Ad5hr-SIV*env/rev* and Ad5hr-SIV*gag* recombinant priming/gp120 boosting regimen. J. Virol. 77, 8354–8365.

Zhao, J., Lou, Y., Pinczewski, J., Malkevitch, N., Aldrich, K., Kalyanaraman, V.S., Venzon, D., Peng, B., Patterson, L.J., Edghill-Smith, Y., Woodward, R., Pavlakis, G.N., Robert-Guroff, M., 2003b. Boosting of SIV-specific immune responses in rhesus macaques by repeated administration of Ad5hr-SIVenv/rev and Ad5hr-SIVgag recombinants. Vaccine 21, 4022–4035.

Zolla-Pazner, S., Lubeck, M., Xu, S., Burda, S., Natuk, R.J., Sinangil, F., Steimer, K., Gallo, R.C., Eichberg, J.W., Matthews, T., Robert-Guroff, M., 1998. Induction of neutralizing antibodies to T-cell line-adapted and primary human immunodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. J. Virol. 72, 1052–1059.